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Evaluation of Genotoxicity and Mutagenic Effects of Vector/DNA Nanocomplexes in Transfected Mesenchymal Stem Cells by Flow Cytometry

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Abstract

In recent years, there has been a great deal of interest in ex-vivo genetic modification of mesenchymal stem cells (MSCs) to meet various biomedical needs. Considering the self-renewal potential of MSCs, it is critically important to ensure that transfection vectors (gene carriers) do not induce genotoxicity because they could theoretically turn a single stem cell into a cancer-initiating cell. Unfortunately, there is currently no reliable, unbiased, and quantitative method to measure genotoxicity (micronuclei formation) of gene carriers directly in transfected MSCs. Consequently, it has not been possible to study the correlation of vectors' physicochemical characteristics with their impact on stem cell genome stability. To address this deficiency, a flow cytometry-based method with a specialized gating protocol was developed that not only measures micronuclei formation, but also determines the mechanism of mutagenesis (i.e., clastogenic vs. aneugenic) of each vector in transfected MSCs. This gating protocol effectively eliminates all interfering signals associated with aggregated nanoparticles (viral and non-viral), exogenous DNA, and apoptotic/necrotic bodies from the micronuclei measurement process. The presented gating protocol for flow cytometry, which is provided as a template, enables investigators in academia, industry and regulatory bodies to rapidly and reliably evaluate the genosafety profiles of gene carriers. The findings of this study also indicate that highly positively charged lipid- and polymeric-based vectors can induce genotoxicity even without manifesting substantial somatic toxicity. Thus, extreme care must be taken before implanting ex-vivo-modified MSCs back into a patient's body.

Graphical abstract

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Conflicts of Interest

The authors declare no conflict of interest.

Keywords

genotoxicity; gene delivery; nanoparticle; stem cells; micronuclei

Introduction

Stem cells have the ability to self-renew and give rise to any cell type in an organism. Because of this unique characteristic, they have been used as a source of donor cells to replace damaged organs [1]. In addition, stem cells have been utilized as a carrier to deliver therapeutic molecules to cancer cells [2, 3]. In these procedures, mesenchymal stem cells (MSCs) are first harvested from donors and then genetically modified ex-vivo to express a variety of bioactive agents. This genetic engineering process usually involves the use of a gene delivery system (vector) to transfect/transduce the MSCs. Many research laboratories prefer to use non-viral vectors to genetically modify MSCs because they are more cost-effective and, in general, safer than viral vectors. Unfortunately, no quantitative or reliable method is available to measure the genetic aberrations introduced in the genome of MSCs after exposure to gene transfer reagents. Considering the self-renewal potential of MSCs, it is very important to ensure that the vectors do not induce genotoxicity because they could theoretically turn a single stem cell into a cancer-initiating cell. More specifically, studying DNA damage is a vital part of evaluating genotoxicity because chromosomal alteration is a significant event during carcinogenesis. In recent years, the need to evaluate the genotoxicity of gene carriers has been highlighted in several published articles [4–8]. In addition, the US Food and Drug Administration and International Conference on Harmonization (ICH), in a recent version of safety reports, recommended that researchers and industries report the genosafety profiles of pharmaceutical formulation ingredients [9].

The micronuclei formation (MN) assay is part of a series of tests that many new products must go through before reaching the market. This in vitro method measures the generation of nuclear blebs and micronuclei in the cytoplasm of interphase cells to estimate a cell's genetic and chromosomal instability upon exposure to the reagents. To perform this assay, cells are exposed to the reagent of interest, stained using the appropriate dyes (e.g., Giemsa or DNA inter-chelating fluorescent dyes), and analyzed by capturing images using a light or fluorescence microscope and scoring micronuclei formation [10]. One of main disadvantages of using this technique, in addition to its quite time- and labor-intensive nature, is that it produces semi-quantitative results. The reason for this is that hundreds to thousands of pictures of the treated cell samples must be first captured and then analyzed to

determine the number of micronuclei in each sample. Because using microscopy to accurately detect and count the number of micronuclei in each picture produces subjective results, the outcome is usually associated with high degrees of variability and uncertainty. The second important shortcoming of the microscopy-based MN assay is its inability to distinguish micronuclei from aggregated particles, and/or apoptotic/necrotic bodies. Furthermore, the current standard in vitro MN assay is performed using either rodent cells, such as L5178Y, CHO, and V79 cells, or human cells, such as TK6 and HepG2 cells. Regrettably, none of these cell lines can realistically represent human MSCs in genotoxicity assessments because they either have a non-human origin (rodent cells) or lack proper natural DNA repair mechanisms (e.g., TK6 and HepG2 cells) [11].

To address the aforementioned deficiencies, *the objective* of this study was to develop a method that can be used to quantitatively analyze micronuclei formation and determine the mechanisms of mutagenesis that occur in transfected MSCs. To achieve the objective, MSCs were cultured in 96-well plates and then transfected with a plasmid encoding green fluorescent protein (pEGFP) using three non-viral gene transfer reagents. Cell transfection was confirmed using fluorescence microscopy, and the percentages of transfected cells and cells showing somatic toxicity were determined concurrently using flow cytometry. To determine the percentage of cells that formed micronuclei, a specialized gating protocol was developed that effectively excluded the interfering signals associated with aggregated nanoparticles (viral and non-viral), apoptotic/necrotic bodies and aggregated green fluorescent proteins from the measurements. Ultimately, a rapid flow cytometry-based method was developed that could be used to simultaneously determine the percentage of micronuclei that formed and the mechanism of DNA damage (i.e., clastogenic vs. aneugenic) that occurred in each transfected MSC population.

Materials and Methods

Preparation of vector/DNA nanocomplexes and zeta potential measurements

Gene transfer reagents, including GeneIn™ (AMSBIO, CA), Lipofectamine® LTX/PLUS™ (Thermo Fisher Scientific Inc.), and jetPRIME® (Polyplus-transfection® SA), were purchased from commercially available sources. The reagents were mixed with pEGFP-C1 (Clontech, Takara Bio USA, Inc.) to form vector/DNA complexes according to each manufacturer's protocol. The size and zeta potential of each vector/DNA nanocomplexes was measured using Zetasizer (Malvern Co., UK). The size measurements were performed by directly measuring 50 µl of the nanocomplexes after they were incubated at room temperature for 10 minutes. To measure zeta potentials, 100 µl of the nanocomplexes were diluted in approximately 600 µl of a low ionic strength solution of 5 mM NaCl. Afterwards, the zeta potential of each sample was measured using Zetasizer. The results of size and zeta measurements are reported as the mean ± S.D. (n=3).

Evaluation of cell transfection/transduction efficiency and somatic toxicity

Human MSCs (adipose-derived) were purchased from Lonza Inc. (Lonza Inc., NJ). They were grown in Lonza's Basal Growth Media for stem cells with Gentamycin, FBS, and L-glutamine (Lonza Inc., NJ), at 37 °C in 5% CO₂ in a humidified atmosphere. The media

were replaced every two days, and the cells were subcultured every four days to keep the cells subconfluent (<60–70%). MSCs were seeded in 96-well plates at a density of 6000 cells per well. After twenty-four hours, the vector/pEGFP complexes were prepared according to the instructions accompanying each vector (i.e., GeneIn™, Lipofectamine® LTX/PLUS™, and jetPRIME®) and used to transfect MSCs. At forty hours post-transfection, GFP expression was qualitatively and quantitatively analyzed using fluorescence microscopy (Olympus Co., PA) and flow cytometry (CytoFlex, Beckman Coulter, MA), respectively.

To transduce MSCs with a viral vector, an Ad5 adenoviral vector carrying the EGFP gene (Ad-GFP) was purchased (Baylor College of Medicine®, TX). MSCs were seeded in 96-well plates, as described above, and incubated with Ad-GFP at a MOI of 5,000 or 50,000. The percentage of transduced cells was determined using flow cytometry, and the data are presented as the mean $\pm$ S.D. (n=4).

To evaluate somatic toxicity, 10 μ L of propidium iodide solution (0.1 μ g/ μ L) was added to each well shortly before FACS analysis. The solutions were then mixed gently and incubated for 5 minutes in the dark. Cell viability (live/dead) was then quantified using flow cytometry.

Measurement of micronuclei formation using flow cytometry

MSCs were seeded at a density of 6000 cells per well in 96-well plates and transfected as mentioned above. At twenty-four hours post-transfection, the cells were harvested and stained using an In Vitro MicroFlow® Kit according to the manufacturer's protocol (Litron Lab., NY) with some modifications, as described here. The cells were detached using Accutase® (Innovative Cell Technologies, Inc., San Diego, CA) and transferred into a 1.5 mL microfuge tube. The cells were then pelleted using centrifugation (600 g, 6 min). The supernatant was gently discarded, and the tube was placed on ice for 20 min. Using the provided 10 $\times$ stock Buffer solution, 200 μ L of 1 $\times$ Buffer mixture was prepared per tube by adding 2% FBS and adjusting the volume with MilliQ water. The cell pellet was then resuspended in 50 μ L of diluted Dye A. The diluted Dye A contained 8 μ g/mL ethidium monoazide (EMA) and was prepared from a stock solution of Dye A by adding a 1 $\times$ Buffer/2% FBS mixture (0.375 μ L of Dye A stock solution per 50 μ L of final volume). The cells resuspended in Dye A were then incubated on ice for an additional 30 minutes while exposed to a fluorescent light that was positioned 10 cm above the samples. Next, 150 μ L of cold 1 $\times$ Buffer/2% FBS solution was added to each tube, and the tubes were gently vortexed and centrifuged (600 g, 6 min). The supernatant was then discarded. Next, the cell pellet was resuspended in 50 μ L of Lysis Buffer 1, which was prepared beforehand by mixing Incomplete Lysis Buffer 1 with RNase (0.57 μ L from the provided stock RNase tube per 50 μ L of Lysis Buffer 1) and Dye B (0.2 μ M, or generally 0.1 μ L of the stock tube per 50 μ L of final mixture volume). After that, the samples were incubated at 37 °C for 3 hours. During this period, they were protected from light and periodically checked under a microscope to ensure complete cell membrane lysis. Immediately afterwards, a solution containing 0.2 μ M Dye B (0.1 μ L of Dye B stock solution per 50 μ L final volume) in Incomplete Lysis buffer 2 was prepared and added to the samples (50 μ L per sample). The mixture was incubated overnight at room temperature while protected from light. The next day, the samples were

gently vortexed, transferred to a 96-well plate, and analyzed by a CytoFlex Flow Cytometer (Beckman Coulter, MA) using an in-house optimized gating protocol for CytoFlex that was based on the guidelines accompanying the In Vitro Microflow® Kit.

To visualize the outcome of the gating protocol, flow cytometry/cell sorting technique (Beckman Coulter MoFlo XDP Cell Sorter, Beckman Coulter, MA) was used to sort the nuclei, aneuploidic nuclei, and micronuclei into separate tubes. The content of each tube was observed under a confocal microscope (Leica TCS SP8) to estimate the sizes of each sorted population.

Identification of centromeres by anti-kinetochore antibody

MSCs were seeded at the density of 1×10^6 cells per 100 mm³ petri dish and transfected with jetPRIME/pEGFP nanocomplexes (equivalent to 10 µg pDNA). The next day, cells were detached and lysed according to the flow cytometry protocol mentioned above. Using flow cytometry/cell sorting technique along with the developed gating protocol the nuclei, aneuploidic nuclei and micronuclei were sorted into separate tubes. The content of each tube was fixed by 4% paraformaldehyde for 15 min, permeabilized by 0.1% Triton-X 100 for 10 min and blocked by 1% BSA in PBS, containing 0.1% Tween, 22.5 mg/ml glycine, for 30 min. Then, primary mouse monoclonal anti-kinetochore antibody (Anti-CENPC, ab50974, Abcam, MA), diluted 1:250 in 1% BSA/PBST, was incubated with the samples, overnight at 4 °C. Samples were washed three times by PBS and incubated with Alexa Fluor® 647-labeled Rat monoclonal (SB74g) anti-Mouse IgG2b gamma chain secondary antibody, (1:1000 dilution, ab172327, Abcam, MA) for one hour at room temperature in the dark. Samples were washed three times and stained by 300 nM DAPI solution for one minute. Finally, 10 µl of each sample was placed on a glass slide and evaluated by Leica TCS SP8 Confocal Microscope (Leica Microsystems GmbH) using 63× objective lens.

Statistical Analysis

The data are obtained from at least three independent repeats (n=3), unless otherwise stated. The data are presented as mean ± standard deviation (S.D.). Statistical comparisons were performed using the student's t-test in between each test and control group. **p* value <0.05 was considered statistically significant.

Results and Discussion

Characterization of gene carriers in terms of size and surface charge

To develop the method, we looked for candidate vectors that could induce genotoxicity and used these as positive controls. For these studies, we selected three commercially available and widely used non-viral transfection reagents (i.e., GeneIn, Lipofectamine LTX/PLUS, and jetPRIME). These vectors were selected because they are known for their ability to condense pDNA into highly positively charged nanoparticles that are suitable for cellular uptake. Therefore, the aforementioned commercial vectors were complexed with pEGFP, resulting in the formation of nanoparticles, and then characterized in terms of size and surface charge. The results of this study demonstrated that all three non-viral vectors produced nanosize particles with highly positive zeta potentials ranging from +40 to +80

mV (Figure 1A and 1B). Because it has previously been shown that nanoparticles with a surface positive charge of more than +20 mV have the potential to induce genetic aberrations in transfected cells [12], we used these vectors to transfect the MSCs and examined their potential to induce the formation of micronuclei.

Preliminary evaluation of micronuclei formation using microscopy

In the past decades, in addition to methods that evaluate the somatic toxicity of gene carriers (e.g., MTT and LDH release assays), methods such as the micronuclei formation (MN) assay has been developed that uses microscopy to measure the genotoxic effects of reagents. Unfortunately, the use of microscopy-based MN assays to evaluate the genotoxicity of gene carriers has faced difficulties due to several technical issues. As a result, the current microscopy-based MN assay is performed using free vectors and in the absence of exogenous nucleotides (e.g., plasmid or viral DNA) because it has difficulty differentiating between micronuclei (endogenous DNA blebs) and exogenous DNA aggregates. This problem stems from the fact that exogenous DNA aggregates, which are picked up by the cells, can remain attached to the cell surface and/or be located inside the cytoplasm. Consequently, they can have the appearance of micronuclei under a microscope and could therefore be incorrectly counted during the MN scoring process. To visually observe the formation of micronuclei in transfected cells and evaluate whether the nanoparticles interfere with the micronuclei measurement process, we followed a well-established method in which we stained the transfected cells with 4, 6 diamidino-2-phenylindole (DAPI) and then evaluated micronuclei formation using microscopy [12]. While the results of this study showed that this assay can be used to detect micronuclei in both untransfected and transfected cells, it was apparent that there was significant ambiguity in our ability to differentiate the micronuclei from the stained nanoparticles and apoptotic bodies (Figure 2). For example, Figure 2A shows the presence of micronuclei in untransfected MSCs which can be measured without difficulty. In Figure 2B, it can be observed that the stained nanoparticle aggregates appear very similar to micronuclei under the microscope. In Figure 2C, the question mark points to an ambiguous subject that could be either aggregated nanoparticles or micronuclei. The same ambiguities are shown in Figures 2D and 2E, in which the incidence of ambiguity increased as the nanoparticle concentration increased.

Next, we quantified the percentage of micronuclei using conventional microscopy-based MN-assay. The results of this experiment revealed its limitations. Figure 3A shows that micronuclei can be identified in each slide without difficulty when the concentration of nanoparticles is low. However, it becomes very difficult to distinguish micronuclei from the nanoparticles when the nanoparticle concentration increases. As result, this method cannot reliably quantify the percentage of micronuclei in transfected cells (Figure 3B). These results indicate that there is a need for an improved method to reliably distinguish micronuclei from aggregated nanoparticles and apoptotic/necrotic bodies.

Evaluation of cell transfection and somatic toxicity

In the next step, we used the three non-viral vectors listed above, carrying different amounts of pEGFP, to transfect MSCs. The transfected cells were analyzed using flow cytometry to simultaneously quantify transfection efficiency and vector-related somatic toxicity. Here, we

used an adenoviral vector (Ad-GFP) as a positive control because adenoviral particles are able to efficiently transfect MSCs. GFP expression indicated that the nanoparticles had effectively reached the nucleus of the transfected cells because DNA transcription occurs in the cell nucleus. The results of this study showed that all three non-viral vectors were capable of transfecting MSCs and that their transfection efficiencies were directly correlated with the amount of pEGFP (Figure 4A, 4B and Supporting Information 2). The adenoviral vector (Ad-GFP) also transfected MSCs with high efficiency. For example, the transfection efficiency of Ad-GFP reached close to 100% at a multiplicity of infection (MOI) of 50,000. It is worth mentioning that we transfected MSCs with Ad-GFP at various MOIs to obtain a range of transfection efficiencies from ~3% to almost 100% (Supporting Information 3) [13]. However, for simplicity we have only shown the results related to transfection efficiencies of Ad-GFP at 5 K and 50 K MOI because the former shows about 50% efficiency (midpoint) and the latter almost 100% (upper limit). Having verified that the vectors could internalize and transfect MSCs, we next evaluated their somatic toxicity.

It is necessary to determine a vector's somatic toxicity profile because highly toxic agents may increase the prevalence of apoptotic and necrotic bodies so much that they would make the genotoxicity assay irrelevant. In general, it is recommended that in a genotoxicity study, the somatic toxicity of an appropriate dose should not exceed 50–55% [14]. It is worth noting that an agent that induces somatic toxicity in more than 55% of the cells may not be worth testing in a genotoxicity screening process. In fact, a genotoxicity study is considered valuable for identifying those reagents that do not induce significant somatic toxicity and are therefore considered superficially safe even though they may have significant hidden genotoxic effects. Keeping this information in mind, we exposed MSCs to escalating amounts of vector/pEGFP complexes in order to determine how much of each vector is needed to induce less than 50% cytotoxicity. The results of brightfield microscopy indicated that MSCs were sensitive to the vectors and that as the concentration of the vectors increased, cell viability decreased (Supporting Information 4). This was demonstrated as cell membrane shrinkage. The flow cytometry-based toxicity assay also revealed significant vector-related toxicity (Figure 4A and 4C). Overall, jetPRIME, which is a polymeric non-viral vector based on PEI (polyethyleneimine), produced cytotoxicity below the 50% threshold when carrying 0.02 µg, 0.05 µg and 0.1 µg of pEGFP. The cytotoxicity data for GeneIn and Lipofectamine showed that all of the tested pDNA amounts below 0.4 µg of pEGFP were acceptable for use in genotoxicity assays because these amounts induced less than 50% somatic toxicity. Interestingly, Ad-GFP, even when used at a high MOI of 50 K, resulted in less than 10% somatic toxicity. Based on these data, we performed the next set of studies to evaluate the efficacy of a flow cytometry-based method for measuring the formation of micronuclei in samples that showed less than 50% somatic toxicity.

Quantification of micronuclei in transfected MSCs by flow cytometry

We transfected the MSCs with the three non-viral vectors and the selected pEGFP concentrations that produced lower than 50% somatic toxicity. At twenty-four hours post-transfection (equivalent to 1–1.5 doubling times), the cells were harvested, stained according to the mentioned protocol and analyzed using flow cytometry. This time-frame for cell harvesting is important because it minimizes the possibility of stress-induced micronuclei

formation, such as overcrowding (high confluency). Next, we developed a specialized gating protocol to measure the percentage of micronuclei in each transfected and untransfected group. The FACS gating protocol is provided as a template that can be used with ease by any investigator to measure genotoxicity (Supporting Information 5).

To analyze data, we selected almost all of the events in a side scatter vs. forward scatter dot plot (Figure 5A–i). Then, the doublet nuclei were discriminated and excluded according to the FITC width vs. FITC area plot (Figure 5A–ii). Next, the SYTOX Green-positive events (Dye B-positive) were selected (Figure 5A–iii). This resulted in two different dot plots that illustrated the nuclei and micronuclei populations and displayed the correct sizes and patterns (Figures 5A–iv and 5A–v). This gating process excluded interfering events, such as smaller fluorescent particles (e.g., GFP aggregates, stained plasmids, or particles). In the next step, the EMA-positive events (Dye A) that originated from dead or late apoptotic cells were excluded (Figure 5A–vi). At this point, the numbers and percentages of micronuclei and nuclei that were released due to the cell wall lysis could be quantified (Figure 5A–vii). The complete lysis of cell walls was confirmed by microscopy (Supporting Information 6). In general, micronuclei were defined as events showing 1/10 to 1/100 of the mean intensity of SYTOX Green fluorescence that was observed in the nuclei of viable (i.e., EMA-negative) cells.

To verify that the events shown in Figure 5 vii are in fact nuclei, aneuploidic nuclei and micronuclei, we used flow cytometry to sort each population into separate tubes and observed the contents under a fluorescent microscope. The results of this experiment illustrated that the sorted particles by the gating protocol are indeed nuclei, aneuploidic nuclei and micronuclei as estimated by their corresponding sizes (Figure 5B).

This gating protocol was utilized to analyze all of the samples. For each sample, at least 1000 EMA-negative nuclei events were counted. The results of this study revealed that the untransfected control group contained less than 1% micronuclei. This result is consistent with data published regarding undamaged cells, indicating that our gating protocol accurately detected the baseline level of micronuclei in untransfected MSCs [14]. As expected, the control group that was transfected with Ad-GFP did not show any significant MN induction even at an extremely high MOI of 50 K (Figure 6). Adenoviruses usually do not induce genotoxicity [15], because they have a non-integrating nature and a negative surface charge. This observation was important because it further validated our flow cytometry gating protocol in two different ways. First, it showed that GFP expression was effectively excluded because the green fluorescence of the expressed protein did not interfere with the SYTOX Green-stained endogenous DNA. If there had been significant overlap in their emission spectra, especially in the cells transfected with Ad-GFP at a MOI of 50 K, the percentage of micronuclei would have reached close to 100%. However, our data show that the percentage of micronuclei in the Ad-GFP-transfected cells was statistically similar to the percentage in the untransfected control, which was lower than 1% (Figure 6). Second, the fact that Ad-GFP did not induce micronuclei at a high MOI also demonstrates that the gating protocol effectively eliminated the DNA-containing viral particles and their aggregates from the measurements without mistaking them as micronuclei. Among the non-viral vectors, only jetPRIME carrying 0.02 µg of pEGFP did

not significantly increase micronuclei formation. Hence, this combination was deemed non-genotoxic ($p>0.05$) (Figure 6). A statistical analysis indicated that the three vectors were considered genotoxic at all other pEGFP concentrations. However, there are no clear FDA or ICH guidelines indicating beyond what fold-increase in micronuclei induction an agent should be considered genotoxic. Nevertheless, a few studies have defined the threshold as a 3-fold increase in MN induction [14, 16]. This means that an agent can be considered genotoxic if it induces micronuclei formation at a rate at least 3-fold higher than that observed in the untreated control group (Figure 6, **dashed line**). We believe that this threshold must be set on a case-by-case basis based on in vivo tests because the three-fold increase threshold may not be appropriate in some cells, such as stem cells. We also observed that all three vectors showed a direct dose-response increase in the amount of MN measured. To determine whether the vector/pEGFP aggregates were effectively excluded from the MN measurements, we manually added very large numbers of vector/pEGFP complexes to the untransfected MSCs immediately before performing flow cytometry. In these experiments, we complexed each non-viral vector with 2.5 μ g or 5 μ g of pEGFP and then added the resulting nanoparticles to microfuge tubes containing untransfected MSCs. This is almost ten times the amount of pEGFP than was used in our transfection studies. If the developed gating protocol worked effectively, we expected to observe that less than 1% of the cells would form micronuclei in each sample regardless of the number of added nanoparticles. Indeed, the results showed that the number of micronuclei in the untransfected MSCs (without nanoparticles) was similar to the number observed in the untransfected MSCs that were spiked with nanoparticles (Supporting Information 7). Overall, our method makes it possible to eliminate all false-positive DNA nanoparticle aggregates from measurements, a task that cannot be accomplished by current semi-quantitative microscopy-based methods. These results clearly show that it is important to perform genotoxicity assays on gene carriers because those that do not show significant cytotoxicity can still be highly genotoxic.

Determining the mechanism of mutagenicity using flow cytometry

The mechanism of action (MOA) for mutagens can be classified as aneugenic or clastogenic. A clastogen is a mutagenic agent that induces chromosome breakage and thereby causes the deletion of chromosome segments, whereas an aneugen induces chromosome loss [17]. Currently, mutagenic mechanisms are determined by using anti-kinetochore antibody staining followed by observation under a microscope. This approach identifies the presence of kinetochore protein in micronuclei associated with the centromeres of chromosomes or its absence from acentric chromosome fragments [18]. Therefore, the interaction of an anti-kinetochore antibody with the micronuclei makes it possible to classify them into kinetochore-positive and kinetochore-negative, specifying their source by aneugenic or clastogenic mechanisms, respectively [19].

Here, we examined the potential use of the developed flow cytometry-based method to determine the mechanism of mutagenicity. An important advantage of the flow cytometry-based method developed here is that it detects the mechanism of mutagenicity more efficiently than the current tedious microscopy-based methods because it determines this mechanism using the data from the same gating protocol. As shown in Figure 6A, the

percentages of nuclei, aneuploidic nuclei, and micronuclei can be determined from flow cytometry dot-plots. The percentage of aneuploidic nuclei reflects aneugenic effects, whereas the percentage of micronuclei reflects the clastogenic effects of a toxic agent. As shown in the dot-plots presented in Figure 6A, the nuclei population is shown in box P5, the aneuploid nuclei in box P7, and the micronuclei in box P6. Therefore, by comparing the pattern of events that appear in P6 and P7, one can determine whether clastogenicity or aneugenicity has occurred. When an increase in the dose of a toxic agent results in an increase in the population of aneuploid nuclei (box P7), the agent is considered aneugenic. Similarly, an agent is considered clastogenic when an increase in its dose results in an increase in the population of micronuclei (box P6). Using the data shown in Figure 6A, we plotted the P6/P7 vs. pDNA dose for each non-viral vector in order to determine the type of mutagenic effect. Mathematically speaking, a positive slope would indicate clastogenicity, whereas a negative slope would indicate aneugenicity. As shown in Figure 7, the effects of all three vectors on the MSC genome were clastogenic rather than aneugenic.

In the past, microscopy-based semi-quantitative methods have demonstrated that highly cationic vectors can induce DNA damage (e.g., crosslinks or breakage) either through direct interaction with DNA or indirectly through generation of reactive oxygen species (single- and double-strand breaks) [20, 21]. The results of this study demonstrate quantitatively that the examined cationic vectors can cause DNA damage through their clastogenic effects.

To validate the outcome of the flow cytometry-based method, we utilized the conventional microscopy-based method to determine the MOA. Therefore, we incubated the sorted nuclei, aneuploidic nuclei and micronuclei with anti-kinetochore antibody to stain the centromeres followed by observation under a confocal microscope. The results of this experiment showed that the anti-kinetochore antibody detected the centromeres in cell nucleus and aneuploidic nuclei but failed to bind to micronuclei indicating their clastogenic origin (Figure 8). This observation is in agreement with the findings of the flow cytometry-based method shown in Figure 7.

Conclusions

The genetic engineering of stem cells is a relatively new field that is aimed at identifying new therapeutic and diagnostic approaches. Because stem cells are fragile in nature, the genomic instability that is induced by common non-viral vectors during the genetic modification process is generally unavoidable. Thus, the current guidelines for cell modifications strongly suggest performing several post-transfection cyto/genosafety tests. Hence, approaches that measure the genotoxicity of every component and transfection vector are gradually becoming standard practice. Overall, in comparison to the traditional methods, the developed flow cytometry method with specialized gating protocol provides efficiency, reliability and cost-effectiveness for measuring the genotoxic effects of vectors in transfected stem cells (Table 1).

Notably, the results of these measurements were obtained without any interference from exogenous DNA (i.e., pDNA and viral DNA), protein aggregates or cell debris. In addition to measuring micronuclei, this method can concurrently determine the mode of mutagenic

activity (e.g., in this study, we evaluated the effects of non-viral and adenoviral vectors on transfected MSCs). The findings of this study also indicate that highly positively charged lipid- and polymeric-based vectors can induce genotoxicity; thus, extreme care must be taken before implanting ex-vivo-modified MSCs back into a patient's body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Significance

There is a great interest in genetic modification of stem cells (SCs) by using vectors for various biomedical needs. Considering the self-renewal potential of SCs, it is essential to ensure that such vectors do not induce genetic aberrations (genotoxicity) because they could theoretically turn a single stem cell into a cancer-initiating cell. Unfortunately, there is currently no reliable method to measure genotoxicity of vectors directly in transfected SCs. To address this deficiency, a specialized flow cytometry-based method was developed that quantitatively analyzed genotoxicity and determined the mechanism of mutagenesis that occurred in transfected SCs during the transfection process. The developed technique will enable scientists to design safer vectors for genetic modification of stem cells.

Figure 1. Characterization of commercial vectors (i.e., jetPRIME, GeneIn and Lipofectamine LTX/PLUS) in terms of size and charge. A) Size analysis of nanoparticles formed through complexation of each vector with 0.5 μ g of pEGFP. The nanoparticles were formed following manufacturers' protocols. B) Surface charge (Zeta potential) analysis of the same nanoparticles.

Figure 2.

Microscopy-based MN assay showing the presence of micronuclei (red arrows), apoptotic and necrotic bodies (green arrows), aggregated nanoparticles (yellow arrows) and ambiguities (question marks). A) Untransfected MSCs; B) Stained vector/pEGFP nanoparticles under the microscope before transfection. C) MSCs transfected with vector/pEGFP complexes carrying 0.2 µg pEGFP; D) MSCs transfected with vector/pEGFP complexes carrying 0.5 µg pEGFP; E) MSCs transfected with vector/pEGFP complexes carrying 0.6 µg pEGFP. The scale bar is 50 µm.

Figure 3. Quantification of micronuclei formation by microscopy. A) Representative microscopy slides of untransfected MSCs (negative control), MSCs transfected with jetPRIME/pEGFP complexes carrying 0.02 µg and 0.1 µg pEGFP, Lipofectamine/pEGFP complexes carrying 0.2 µg and 0.6 µg pEGFP, and GeneIn/pEGFP complexes carrying 0.2 µg and 0.6 µg pEGFP. The cells were stained with DAPI and then observed under a fluorescent microscope (40× objective lens). Scale bar is 50 µm. B) Quantification of micronuclei formation by visually observing and counting the number of micronuclei in each untransfected and transfected group. Each data point is an average of micronuclei in at least 10 slides. For details, please see Supporting Information 1. ND: not determined.

Figure 4.

Evaluation of the transfection efficiencies and somatic toxicities of adenovirus (Ad-GFP) and vector/pEGFP nanocomplexes. A) Representative flow cytometry dot-plots showing the percentages of transfected and live/dead cells. B) Bar chart summarizing the percentage of GFP-positive cells in the total number of transfected MSCs when non-viral vectors and Ad-GFP were used. The transfection efficiencies of Ad-GFP at 5K and 50K are included as controls since they provide almost 50% (midpoint) and 100% efficiencies (upper limit). C) Bar chart summarizing the percentage of live cells in the total number of transfected MSCs. The dashed line crossing at the 50th percentile indicates the acceptable somatic toxicity threshold for genotoxicity assays.

Figure 5.

A) The gating protocol used to measure micronuclei formation. In all of the acquired events shown in panel i, the doublets were gated out (panel ii), and the SYTOX Green-positive events were then selected (panel iii). pDNA aggregates, cell debris, and GFP aggregates were then excluded using the gatings shown in panels iv and v. Late apoptotic and necrotic bodies were removed using the gating shown in panel vi. Finally, the percentage of micronuclei was measured according to panel vii. Thus, the final micronuclei were SYTOX Green-positive and EMA-negative. B) Fluorescent microscopy images of SYTOX Green-stained and sorted nuclei, aneuploidic nuclei and micronuclei from panel A-vii. The scale bar is 50 μ m (63 $\times$ objective lens).

Figure 6.

Evaluation of micronuclei induction after transfection of MSCs with non-viral and viral vectors. A) Representative flow cytometry dot-plots of transfected MSCs showing the percentages of micronuclei (box P6), aneuploidic nuclei (box P7) and nuclei (box P5) in each group. B) Corresponding bar chart summarizing the percentages of micronuclei in transfected MSCs. The dashed line demonstrates the threshold at which there is a more than 3-fold increase in micronuclei induction over the results observed in the untreated MSCs. * indicates significant statistical difference in comparison to control ($p < 0.05$).

Figure 7.

Correlation between pDNA dose and the ratio of micronuclei to aneuploid nuclei in transfected MSCs as measured by flow cytometry. After treating the MSCs with nanoparticles that formed through complexing of different amounts of pEGFP (μg) with (A) jetPRIME, (B) Lipofectamine, and (C) GeneIn, the gating protocol was utilized to determine P6 (micronuclei) and P7 (aneuploidic nuclei). The positive correlations shown in these graphs indicate that each of the three non-viral vectors tested here has a clastogenic mode of activity.

Figure 8.

Confocal microscopy images of the sorted nuclei, aneuploidic nuclei and micronuclei after labeling with anti-kinetochore antibody. Upper panels show the confocal microscopy images of samples stained with DAPI, whereas the middle panels show the Alexa Fluor 647 labeled samples indicating the presence/absence of centromeres. The lower panels (Merged images) show that nuclei and aneuploidic nuclei samples were centromere-positive, but micronuclei sample was centromere-negative.

Summary of current microscopy-based and flow cytometry-based methods used for the measurement of micronuclei formation in MSCs transfected with non-viral and viral vectors.

Table 1

Methods	Data Analysis	Application in MN Measurement	Detection of MOA	High throughput	Refs.
Microscopy	Semi-Quantitative	Yes, but limited	Tedious, Needs Ab	No	[10–12, 19]
FACS-STD	Quantitative	Not Shown	Not Shown	Yes	[14, 16]
FACS-SP _{SC}	Quantitative	Yes- in stem cells	Simple Doesn't need Ab	Yes	---

MOA: mode of action, MN: micronuclei, Ab: antibody against centromere, FACS: fluorescence-activated cell sorting, STD: standard method, SP_{SC}: Specialized method for stem cells (SC).